

# Involvement of the Eukaryote-Like Kinase-Phosphatase System and a Protein That Interacts with Penicillin-Binding Protein 5 in Emergence of Cephalosporin Resistance in Cephalosporin-Sensitive Class A Penicillin-Binding Protein Mutants in *Enterococcus faecium*

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**ABSTRACT** The intrinsic resistance of *Enterococcus faecium* to ceftriaxone and cefepime (here referred to as “cephalosporins”) is reliant on the presence of class A penicillin-binding proteins (Pbbs) PbpF and PonA. Mutants lacking these Pbbs exhibit cephalosporin susceptibility that is reversible by exposure to penicillin and by selection on cephalosporin-containing medium. We selected two cephalosporin-resistant mutants (Cro1 and Cro2) of class A Pbp-deficient *E. faecium* CV598. Genome analysis revealed changes in the serine-threonine kinase Stk in Cro1 and a truncation in the associated phosphatase StpA in Cro2 whose respective involvements in resistance were confirmed in separate complementation experiments. In an additional effort to identify proteins linked to cephalosporin resistance, we performed tandem affinity purification using Pbp5 as bait in penicillin-exposed *E. faecium*; these experiments yielded a protein designated Pbp5-associated protein (P<sub>5</sub>AP). Transcription of the P<sub>5</sub>AP gene was increased after exposure to penicillin in wild-type strains and in Cro2 and suppressed in Cro2 complemented with the wild-type *stpA*. Transformation of class A Pbp-deficient strains with the plasmid-carried P<sub>5</sub>AP gene conferred cephalosporin resistance. These data suggest that Pbp5-associated cephalosporin resistance in *E. faecium* devoid of typical class A Pbbs is related to the presence of P<sub>5</sub>AP, whose expression is influenced by the activity of the serine-threonine phosphatase/kinase system.

**IMPORTANCE**  $\beta$ -Lactam antibiotics remain our most effective therapies against susceptible Gram-positive bacteria. The intrinsic resistance of *Enterococcus faecium* to  $\beta$ -lactams, particularly to cephalosporins, therefore represents a major limitation of therapy. Although the primary mechanism of resistance to  $\beta$ -lactams in *E. faecium* is the presence of low-affinity monofunctional transpeptidase (class B) penicillin-binding protein Pbp5, the interaction of Pbp5 with other proteins is fundamental to maintain a resistant phenotype. The present work identifies a novel, previously uncharacterized, protein that interacts with Pbp5, whose expression increases in conjunction with stimuli that increase resistance to cephalosporins, and that confers increased resistance to cephalosporins when overexpressed. P<sub>5</sub>AP may represent a promising new target, inhibition of which could restore cephalosporin susceptibility to *E. faecium*.

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*Enterococcus faecalis* and *Enterococcus faecium* differ from the closely related streptococci by virtue of their intrinsic resistance to cephalosporin antibiotics and their reduced susceptibility to penicillins. This resistance has been attributed to the expression of low-affinity class B penicillin-binding protein (Pbp) Pbp5 (1), a transpeptidase that must work in coordination with class A Pbp glycosyltransferases to be able to synthesize peptidoglycan in the presence of cephalosporin antibiotics (2). *E. faecium* strains in which Pbp5 has been deleted are susceptible to  $\beta$ -lactam antibiotics, including cephalosporins (3).

Several other loci are involved in cephalosporin resistance, including two of the three identified class A Pbbs (PonA and PbpF) (2, 4), the CroRS system (in *E. faecalis*) (5), the eukaryote-like kinase/phosphatase systems (Stk/StpA in *E. faecium* and IreK/IreP in *E. faecalis*) (6–8), and the early cell wall synthesis enzyme (MurA) (9). Ampicillin resistance has been achieved in an *E. faecium* strain devoid of Pbp5 (10) through the activity of a penicillin-/cephalosporin-insensitive L,D-transpeptidase and a D,D-carboxypeptidase where the phenotype is augmented by mutations that reduce the activity of StpA (11).

Eukaryote-like serine-threonine kinase/phosphatase systems

are widespread in bacteria and have been implicated in many different cellular processes, including cell wall synthesis, cell division, and susceptibility to cell wall-active antibiotics (12). The effects of protein phosphorylation are numerous and include changes in regulation of transcription, protein activity, and protein-protein interactions.

The presence of class A Pbp PonA or PbpF was required for cephalosporin resistance in *E. faecalis* (4). Our own experiments performed in *E. faecium* yielded similar results (2), in which deletion of the *E. faecium* *pbpF* and *ponA* resulted in a strain that became susceptible to certain cephalosporins (ceftriaxone and cefepime, here referred to simply as cephalosporins) but not others (cefazolin and cefoxitin). These changes had no detectable impact on susceptibility to ampicillin. In addition, we found that phenotypic resistance to cephalosporins in these class A Pbp mutants could be induced by exposure to penicillin and could be readily selected in low-frequency mutants by growing large inocula on ceftriaxone-containing plates.

The present experiments were undertaken in an effort to understand the mechanisms of *E. faecium* cephalosporin susceptibility and resistance in the absence of class A Pbps. Our prior work indicated that cephalosporin resistance could be induced in class A Pbp mutants by exposure to penicillin, a phenotype that was reversible with removal of penicillin (2). To investigate the mechanisms of this phenotype, we examined changes in transcription among class A Pbp mutants after growth in the presence or absence of penicillin. We also observed that mutants stably resistant to cephalosporins could be selected from the class A Pbp-deficient strains on cephalosporin-containing media. We performed whole-genome sequencing in the generated cephalosporin-resistant mutants to identify changes linked to the resistant phenotype in the hopes that we might identify common pathways to cephalosporin resistance under the different circumstances. Finally, we used Pbp5 as bait in tandem affinity purification (TAP) experiments designed to identify proteins interacting with Pbp5 under conditions resulting in the cephalosporin-resistant phenotype (exposure to penicillin). We report the identification of a protein (Pbp5-associated protein [P<sub>5</sub>AP]) found in association with Pbp5 whose expression is increased in a cephalosporin-resistant mutant of the StpA-Stk eukaryote-like serine-threonine kinase/phosphatase system and by exposure to penicillin. In addition, overexpression of P<sub>5</sub>AP in a class A Pbp *E. faecium* mutant resulted in an increase in cephalosporin MIC, confirming its importance in phenotypic cephalosporin resistance in *E. faecium*.

## RESULTS

**Selection and characterization of cephalosporin-resistant mutants.** The strains and plasmids used in these experiments are listed in Table 1. *E. faecium* CV598 ( $\Delta pbpF \Delta ponA \Delta pbpZ$  Chl<sup>r</sup>) is a cephalosporin-susceptible mutant in which all three of the known class A Pbp genes have been deleted, with the chloramphenicol resistance (*cat*) gene used to replace *pbpZ* left in place (2). A 0.5 McFarland standard was made in brain heart infusion (BHI) from a fresh overnight plate, and 100  $\mu$ l of each was inoculated onto BHI plates with a ceftriaxone disc. After 2 to 3 days, colonies appeared within the ceftriaxone zone and were recovered, purified for single colonies, and tested for susceptibility to ceftriaxone. One such ceftriaxone-resistant colony was chosen and sent for whole-genome sequencing. The mutations found in the resistant mutant (Cro1; ceftriaxone MIC, 250  $\mu$ g/ml [Table 2]) are listed in Ta-

ble S1 in the supplemental material. None of the mutations were in *pbp5*. The most interesting mutation found in Cro1 was in *stk* (*ireK* in *E. faecalis*), which has been identified as involved in the cephalosporin resistance phenotype of *E. faecalis* (6). The mutation introduced a premature stop codon 33 amino acids (aa) from the C terminus of the protein, in a region that extends beyond the PASTA domains typical of the extracellular portion of eukaryote-like serine-threonine kinases and is composed predominantly of serines (see Fig. S1 in the supplemental material). A search of the Swiss-Prot database yielded 34 *E. faecium* sequences, all with the serine tail. Similar serine-rich tails were present on *stk* homologues in *Enterococcus durans*, *Enterococcus casseliflavus*, *Enterococcus mundtii*, *Enterococcus flavescens*, and *Peptoclostridium difficile* Y384.

*E. faecium* CV558 ( $\Delta pbpF \Delta ponA$  Cm<sup>r</sup>) and CV571 ( $\Delta pbpF \Delta ponA$ ) are double class A Pbp mutants with the same cephalosporin-susceptible phenotype as CV598. They grow more robustly than CV598 and are more amenable to genetic manipulation and so were used for most cloning and complementation experiments. We attempted to delete *stk* from CV571 without success. *stk* exists in an operon downstream of and cotranscribed with its cognate phosphatase (*stpA*). Prior work in *E. faecalis* suggested that cloning the two genes together yields better results than either alone (C. Kristich, personal communication). We amplified and cloned both the native and mutant *stpA-stk* coding sequences into the shuttle plasmid pTCV-lac under the control of a rhamnose-inducible promoter and reintroduced both into *E. faecium* CV558. There was no change in ceftriaxone MIC associated with introduction of the native coding sequence into CV558. However, introduction of the mutant *stk* with the native *stpA* was associated with rhamnose-inducible resistance to ceftriaxone (Table 2), confirming the association of this mutation with the cephalosporin-resistant phenotype. This indicates that the mutated gene is dominant over the wild type and suggests that the mutation results in an augmentation of kinase activity. Western blot analysis of crude cellular extracts from CV598 and the Cro1 mutant with anti-Stk antibody confirmed that a protein of smaller size was produced in the Cro1 mutant (Fig. 1A).

In an effort to determine whether mutations in *stpA-stk* represent a common pathway to cephalosporin resistance in our class A Pbp mutants, we selected another round of ceftriaxone-resistant mutants of CV598. Two of these were purified and passaged as before, and *stpA* and *stk* were amplified and sequenced. In one of these two mutants (Cro2; ceftriaxone MIC, 1,000  $\mu$ g/ml [Table 2]), we detected a T-to-A change at position 369 of *stpA*, which converts a tyrosine to a stop codon after 123 amino acids of a protein predicted to be 246 aa in length, resulting in a truncated and presumably nonfunctional protein. Transformation of the Cro2 mutant with the native *stpA-stk* resulted in a return of cephalosporin susceptibility (see Fig. S2 in the supplemental material), suggesting that the mutation resulted in a nonfunctional protein and that the phosphatase activity is associated with cephalosporin susceptibility in these strains. Western blot analysis of crude cellular extracts from the Cro2 mutant (Fig. 1A) did not identify StpA, which, given the Cro2 mutation, would be expected to be half the size of the full protein and potentially unstable. Surprisingly, only a very faint band was observed corresponding to the expected location for the Stk band, despite quantities of message comparable to those found in the Cro2 mutant by reverse transcription-quantitative PCR (RT-qPCR) (Fig. 1B), suggesting

TABLE 1 Strains and plasmids used in this study<sup>a</sup>

| Strain or plasmid                   | Relevant phenotype   | Resistance trait(s)   | Reference or source |
|-------------------------------------|--|---|---------------------|
| <i>Enterococcus faecium</i> strains |  |   |                     |
| D344R                               | Wild type, penicillin and ceftriaxone resistant  | Pen <sup>r</sup> Cro <sup>r</sup> Chl <sup>s</sup>  | 3                   |
| CV598                               | $\Delta$ <i>pbpF</i> $\Delta$ <i>pbpZ</i> $\Delta$ <i>ponA</i> , ceftriaxone sensitive, derived from D344R, <i>cat</i> replacing <i>pbpZ</i> | Pen <sup>r</sup> Cro <sup>s</sup> Chl <sup>r</sup> Kan <sup>s</sup>                                   | 2                   |
| CV558                               | $\Delta$ <i>pbpF</i> $\Delta$ <i>ponA</i> , ceftriaxone sensitive, derived from D344R; CAT replacing <i>pbpF</i> gene                        | Pen <sup>r</sup> Cro <sup>s</sup> Chl <sup>r</sup> Kan <sup>s</sup>                                   | 2                   |
| CV571                               | $\Delta$ <i>pbpF</i> $\Delta$ <i>ponA</i> , ceftriaxone sensitive derived from CV558; CAT-deleted chromosomal insertion                      | Pen <sup>r</sup> Cro <sup>s</sup> Chl <sup>s</sup> Kan <sup>s</sup>                                   | 2                   |
| D344S                               | Spontaneous $\Delta$ <i>pbp5</i> from D344R  | Pen <sup>s</sup> Cro <sup>s</sup> Chl <sup>s</sup> Kan <sup>s</sup> Rif <sup>r</sup> Fus <sup>r</sup> | 3                   |
| Cro1                                | Spontaneous ceftriaxone-resistant isolate from CV598   | Pen <sup>r</sup> Cro <sup>r</sup> Chl <sup>r</sup> Kan <sup>s</sup>                                   | This study          |
| Cro2                                | Spontaneous ceftriaxone-resistant isolate from CV598   | Pen <sup>r</sup> Cro <sup>r</sup> Chl <sup>r</sup> Kan <sup>s</sup>                                   | This study          |
| LS002                               | CV571 carrying pRIH011, with Pbp5 fused to TAP peptides  | Pen <sup>r</sup> Cro <sup>s</sup> Chl <sup>s</sup> Kan <sup>r</sup>                                   | This study          |
| LS030                               | CV558 carrying pRIH39, with WT <i>stpA</i> - <i>stk</i> alleles under control of rhamnose promoter   | Pen <sup>r</sup> Cro <sup>s</sup> Chl <sup>r</sup> Kan <sup>r</sup>                                   | This study          |
| LS031                               | CV558 carrying pRIH36, with WT <i>stpA</i> and mutant <i>stk</i> under control of rhamnose promoter  | Pen <sup>r</sup> Cro <sup>r</sup> Chl <sup>r</sup> Kan <sup>r</sup>                                   | This study          |
| LS017                               | D344S carrying pRIH36, with WT <i>stpA</i> and mutant <i>stk</i> genes under control of rhamnose promoter                                    | Pen <sup>s</sup> Cro <sup>s</sup> Chl <sup>s</sup> Kan <sup>r</sup>                                   | This study          |
| LS106                               | CRO2 carrying pRIH39, with WT <i>stpA</i> - <i>stk</i> alleles under control of rhamnose promoter  | Pen <sup>r</sup> Cro <sup>s</sup> Chl <sup>r</sup> Kan <sup>r</sup>                                   | This study          |
| LS107                               | CV571 carrying pRIH115, with <i>pbp5</i> promoter US of TAP peptides   | Pen <sup>r</sup> Cro <sup>s</sup> Chl <sup>s</sup> Kan <sup>r</sup>                                   | This study          |
| LS108                               | CV571 carrying pRIH113, with P <sub>5</sub> AP under control of rhamnose promoter  | Pen <sup>r</sup> Cro <sup>s</sup> Chl <sup>s</sup> Kan <sup>r</sup>                                   | This study          |
| Plasmids                            |  |   |                     |
| pACYC184                            | Low-copy-number cloning vector for growth in <i>E. coli</i>  | Tet <sup>r</sup> Chl <sup>r</sup>   |                     |
| pTCV-lac                            | Conjugative <i>E. coli</i> - <i>E. faecium</i> shuttle vector  | Erm <sup>r</sup> Kan <sup>r</sup>   | 20                  |
| pBS1479                             | C-terminal epitope tag consisting of a calmodulin binding peptide and a protein A moiety separated by a TEV cleavage site                    | Amp <sup>r</sup>  | 22                  |
| pCJK96                              | <i>E. coli</i> - <i>E. faecalis</i> shuttle plasmid containing rhamnose-inducible promoter and regulator from <i>E. faecalis</i>             | Erm <sup>r</sup>  | 7                   |
| pRIH11                              | <i>pbp5</i> -TAP tag fusion in the SmaI site of pTCV-lac   | Erm <sup>r</sup> Kan <sup>r</sup>   | This study          |
| pRIH39                              | Rhamnose promoter and regulator from <i>E. faecalis</i> and WT <i>stpA</i> - <i>stk</i> alleles in SmaI site of pTCV-lac                     | Erm <sup>r</sup> Kan <sup>r</sup>   | This study          |
| pRIH36                              | Rhamnose promoter and regulator from <i>E. faecalis</i> and Cro1 <i>stpA</i> - <i>stk</i> alleles in SmaI site of pTCV-lac                   | Erm <sup>r</sup> Kan <sup>r</sup>   | This study          |
| pRIH115                             | <i>pbp5</i> promoter and TAP tag in SmaI site of shuttle vector pTCV-lac   | Erm <sup>r</sup> Kan <sup>r</sup>   | This study          |
| pRIH113                             | Rhamnose promoter and regulator from pCJK96 US of P <sub>5</sub> AP coding sequence from D344R, in SalI sites of pTCV-lac                    | Kan <sup>r</sup>  | This study          |

<sup>a</sup> Abbreviations: CAT, chloramphenicol acetyltransferase; WT, wild type; US, upstream; Amp, ampicillin; Chl, chloramphenicol; Cro, ceftriaxone; Erm, erythromycin; Fus, fusidic acid; Kan, kanamycin; Pen, penicillin; Rif, rifampin; Tet, tetracycline.

that translation of the kinase may be dependent upon full translation of the phosphatase. A comparison of phosphorylated proteins by phosphoprotein gel staining of SDS-PAGE-separated proteins from crude cellular extracts of the Cro1 and Cro2 mutants and CV598 with or without penicillin exposure showed minor differences in both the degree of phosphorylation and the appearance of newly phosphorylated proteins between the strains (Fig. 1C). SYPRO Ruby was used as a poststain to confirm equivalent amounts of total protein loaded and gave a snapshot of the relative phosphorylation state differences compared to the phosphorylated protein profiles (Fig. 1D).

**Proteins associated with Pbp5.** Based on the assumption that an unidentified glycosyltransferase might be physically associated with Pbp5 during the process of peptidoglycan synthesis in the

double and triple class A Pbp mutants, we analyzed proteins associated with Pbp5 under conditions that confer resistance to cephalosporins. We used the tandem affinity purification (TAP) technique (13) with Pbp5 as the bait protein to isolate, and mass spectrometry to identify, Pbp5-associated proteins after growth of CV571 either with or without exposure to penicillin (10  $\mu$ g/ml). Proteins associated with the TAP tag without Pbp5 were also analyzed to assess background contaminants. A list of the associated proteins is shown in Table S2A in the supplemental material. As expected, peptides derived from Pbp5 were most frequently identified. Also identified in association with Pbp5 and at a higher frequency when cells were incubated with penicillin was a protein of unknown function that we have designated P<sub>5</sub>AP (Pbp5-associated protein) (EDAG\_01115 [protein identifier {ID}]

**TABLE 2** MICs of different antibiotics against cephalosporin-susceptible *E. faecium* CV598 ( $\Delta pbpF \Delta pona \Delta pbpZ$ ) and two cephalosporin-resistant mutants, Cro1 and Cro2, and *E. faecium* CV558 ( $\Delta pona \Delta pbpF$ ) alone or carrying cloned versions of *stpA-stk* from D344R or from the Cro1 mutant in which the terminal portion of Stk has been deleted<sup>a</sup>

| Strain (genotype)  | Plasmid                        | Growth condition | MIC ( $\mu$ g/ml) of drug: |      |                   |      |       |
|--|--------------------------------|------------------|----------------------------|------|-------------------|------|-------|
|  |                                |                  | PenG                       | Amp  | Cro               | Van  | Spec  |
| CV598 ( $\Delta pbpF \Delta pona \Delta pbpZ$ )                  | None                           | Uninduced        | 50                         | 25   | 1.56              | ND   | ND    |
| Cro1 ( $\Delta pbpF \Delta pona \Delta pbpZ$ <i>stk</i> mutant)  | None                           | Uninduced        | 50                         | 25   | 250               | ND   | ND    |
| Cro2 ( $\Delta pbpF \Delta pona \Delta pbpZ$ <i>stpA</i> mutant) | None                           | Uninduced        | 50                         | 25   | >1,000            | ND   | ND    |
| CV558 ( $\Delta pbpF \Delta pona$ )                              | None                           | Uninduced        | 100                        | 50   | 3.13              | 0.78 | 31.25 |
| LS030 ( $\Delta pbpF \Delta pona$ )                              | pRIH39 ( <i>stpA-stk</i> WT)   | Uninduced        | 50                         | 50   | 6.25              | 0.2  | 31.25 |
| LS030 ( $\Delta pbpF \Delta pona$ )                              | pRIH39 ( <i>stpA-stk</i> WT)   | Induced          | 25                         | 50   | 3.13              | 0.2  | 31.25 |
| LS031 ( $\Delta pbpF \Delta pona$ )                              | pRIH36 ( <i>stpA-stk</i> Cro1) | Uninduced        | 100                        | >100 | >100 <sup>b</sup> | 1.56 | 31.25 |
| LS031 ( $\Delta pbpF \Delta pona$ )                              | pRIH36 ( <i>stpA-stk</i> Cro1) | Induced          | 100                        | 100  | >100              | 0.78 | 15.63 |

<sup>a</sup> Abbreviations: WT, wild type; PenG, penicillin; Amp, ampicillin; Cro, ceftriaxone; Van, vancomycin; Spec, spectinomycin; ND, not done.

<sup>b</sup> Promoter does not completely suppress transcription.

EFD09967 from the genome of *E. faecium* D344SRF]) referred to in the mass spectrometry data set as H8LA73\_H8LA73\_ENTFU from the *E. faecium* strain Aus0004 closed genome used as a reference. Peptides from this protein predominated in penicillin-treated cells, were identified with considerably lower frequency in the absence of penicillin, and were not identified at all in the purified protein complexes from the TAP tag without Pbp5 in the absence of penicillin (see Table S2B). The conditions under which P<sub>5</sub>AP is found with greater frequency are the conditions under which greater levels of cephalosporin resistance are achieved.

All 244 available *E. faecium* genome sequences in the EnsemblBacteria database contained P<sub>5</sub>AP, suggesting that it is part of the *E. faecium* core genome. The predicted P<sub>5</sub>AP protein is 522 amino acids in length and contains the domains of unknown function DUF4097 (IPR025164) and DUF4098 (IPR025386); these domains are conserved in all identified homologues of P<sub>5</sub>AP in different *Enterococcus* species. Additionally, we identified proteins with both DUF4097 and DUF4098 domains in other *Lactobacillales*, including *Lactobacillus*, *Aerococcus*, and *Carnobacterium*. Interestingly, the sequences retrieved from *Leuconostoc*, *Streptococcus*, and *Lactococcus* are highly divergent (Fig. 2; see also Fig. S3 and Table S3 in the supplemental material) and do not cluster with other *Lactobacillales*. In more distantly related *Firmicutes*, we identified DUF4097-DUF4098-containing proteins in the *Bacillales*, including *Bacillus*, *Listeria*, and *Staphylococcus* and in the more distant *Clostridia*.

Microarray experiments indicated that the P<sub>5</sub>AP coding sequence was expressed at higher levels in CV571 after penicillin exposure (see Table S4 in the supplemental material), a result that was confirmed by RT-PCR (Fig. 3A). Expression of the P<sub>5</sub>AP coding sequence was not significantly increased in the Cro1 mutant but was increased 2- to 10-fold in the Cro2 mutant (Fig. 3B). Expression was also increased 2-fold in CV598 after penicillin exposure (Fig. 3B), as well as in clinical isolates C68 (4.8-fold) and D344R (2.8-fold) after exposure to penicillin. Expression of P<sub>5</sub>AP was not increased in the Pbp5<sup>-</sup> strain D344SRF (data not shown), suggesting that interaction of penicillin with Pbp5 may be the event leading to the increased expression. Transformation of Cro2 with a plasmid expressing wild-type *stpA-stk* resulted in a substantial reduction in P<sub>5</sub>AP gene expression (Fig. 3C). Transformation of CV571 by the P<sub>5</sub>AP coding sequence cloned under the control of a rhamnose-inducible promoter revealed a rhamnose-induced decrease in susceptibility to ceftriaxone (Table 3). Interestingly,

rhamnose induction was also associated with a 16-fold reduction in susceptibility to ampicillin.

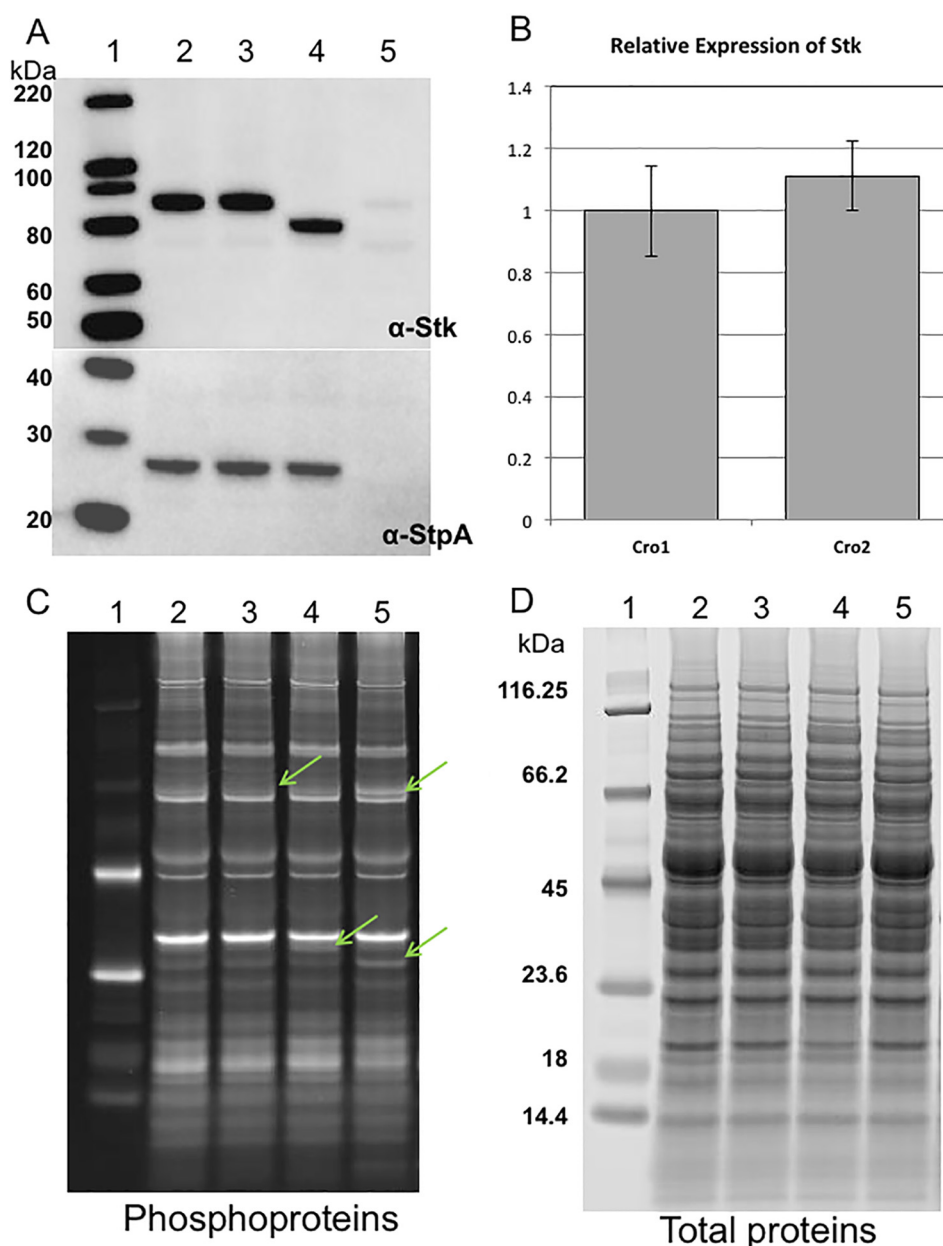
## DISCUSSION

Eukaryote-like serine-threonine kinases are ubiquitous in Gram-positive bacteria and have been shown to be involved in a variety of cellular functions, including virulence, colonization ability, cell envelope biosynthesis, and antimicrobial resistance, among others (12). Our findings that *stpA* and *stk* are involved in cephalosporin resistance in *E. faecium* are expected given the results of prior studies but offer further insight into the function of these genes in this species. Kristich and colleagues (6, 7) have established that the orthologue of Stk in *E. faecalis* (IreK) is required for resistance to cephalosporins and is also involved in colonizing ability in a mouse gastrointestinal model. They also established that IreK is a self-phosphorylating kinase and that IreP works as a phosphatase to modulate the activity of IreK in promoting resistance to cephalosporins, at least in part by serine and threonine dephosphorylation in the IreK active site. Under the conditions of their assay, they found no evidence that IreK phosphorylated IreP. They have identified a single additional target for IreK phosphorylation, designated IreB (14), but there is as yet no defined function for this protein.

A previous study (8) found an impairment of function by a mutation in *stpA* to be associated with modestly increased levels of resistance to ampicillin in *E. faecium* D344S, a strain that is cephalosporin and ampicillin susceptible through spontaneous deletion of 160 kb of genomic DNA that included *pbp5* (15). High-level resistance to ampicillin was achieved in this strain through a series of mutations leading to predominant synthesis of peptidoglycan by an L,D-transpeptidase whose tetrapeptide substrate was created by the activity of a D,D-carboxypeptidase that is variably present in different *E. faecium* strains. Mutations leading to decreased function of the StpA or increased expression of the D,D-carboxypeptidase alone resulted in only modest increases in ampicillin resistance, but the combination of the two mutations led to very high levels of resistance. Their studies also established that StpA, similar to IreP, is capable of dephosphorylating its cognate kinase Stk.

Our data indicate that, as in *E. faecalis*, cephalosporin resistance in *E. faecium* expressing low-affinity Pbp5 is reciprocally controlled by the phosphatase and kinase activities of StpA and Stk. Eukaryote-like serine-threonine kinases have a characteristic

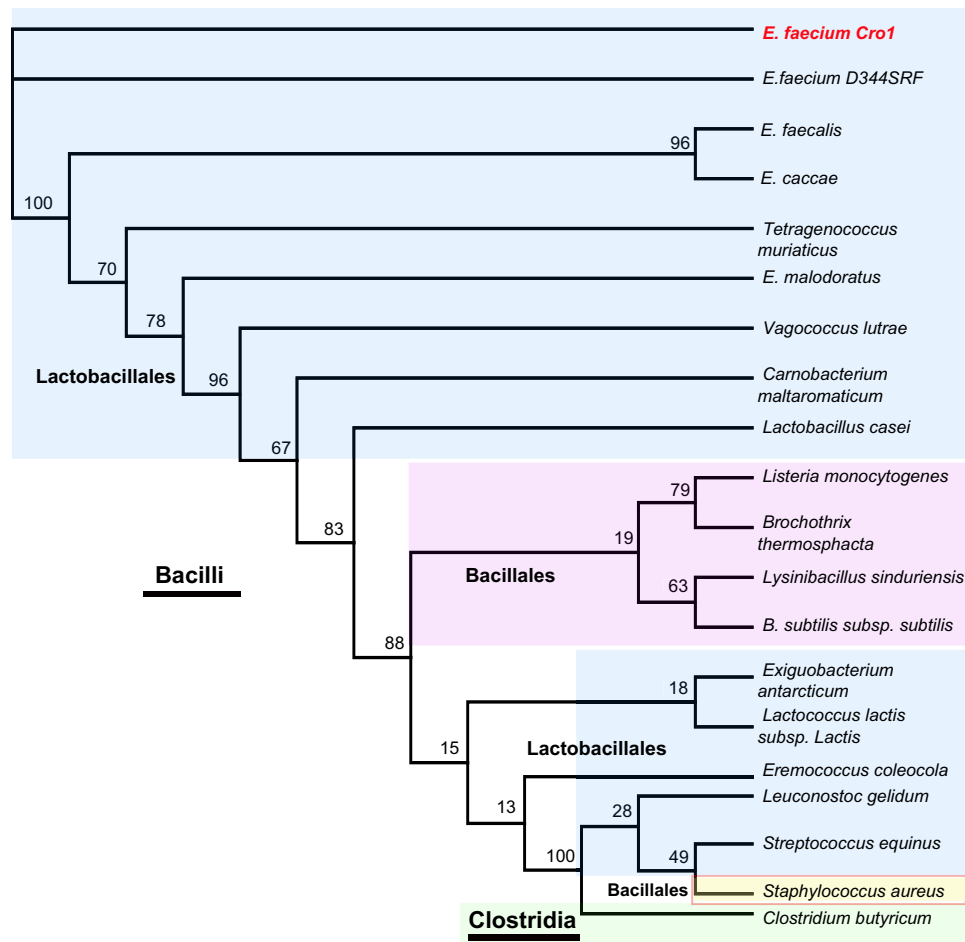




**FIG 1** Expression of Stk and StpA in CV598, CV598 plus penicillin (10  $\mu$ g/ml), Cro1 mutant, and Cro2 mutant from whole-cell lysates at mid-exponential growth phase. (A) (Top) Western blot analysis of CV598 (lane 2), CV598 plus penicillin (lane 3), Cro1 mutant (lane 4), and Cro2 mutant (lane 5) using polyclonal antibody to Stk. The smaller size of the band in lane 4 confirms the truncation of the terminus of Stk. Only a very faint Stk band appears for the Cro2 mutant, suggesting that translation of the full StpA and that of Stk are linked, possibly through a translational coupling mechanism. (Bottom) Western blot analysis of whole-cell lysates from strains listed in the upper panel using a polyclonal antibody to StpA. As expected, there is no band in the expected size range of StpA for the Cro2 mutant, whose predicted amino acid sequence is truncated approximately halfway through the protein. (B) Relative expression of Stk in the Cro1 and Cro2 mutants. Expression levels of 16S rRNA and *tufA* were used as references. Error bars indicate the standard error of the mean for biological duplicates. Results are representative of two independent experiments. (C) Phosphoprotein analysis, by phosphoprotein gel-stained SDS-PAGE of whole-cell lysates of strains depicted in panel A. Arrows indicate minor differences in the strains. (D) Total protein analysis of whole-cell lysates of strains depicted in panel A, revealing equivalent amounts of total protein in the preparations.

structure in which the kinase domain is cytoplasmic and connected to an external series of PASTA (PBP- and serine/threonine kinase-associated) domains thought to interact with nascent peptidoglycan in a manner that detects modifications potentially deleterious to the bacterium (12). The structure of *E. faecium* Stk differs from most others in that the PASTA domains are con-

nected to a “tail” that is composed predominantly of serines. The mutation observed in Stk in Cro1 occurred in this tail end of the molecule, truncating the serine string by 33 amino acids, as evidenced by the Western blot showing a smaller protein in the mutant strain (Fig. 1A). We presume that this mutation leads to an increase in Stk activity, since cloning and expressing the *stpA-stk*

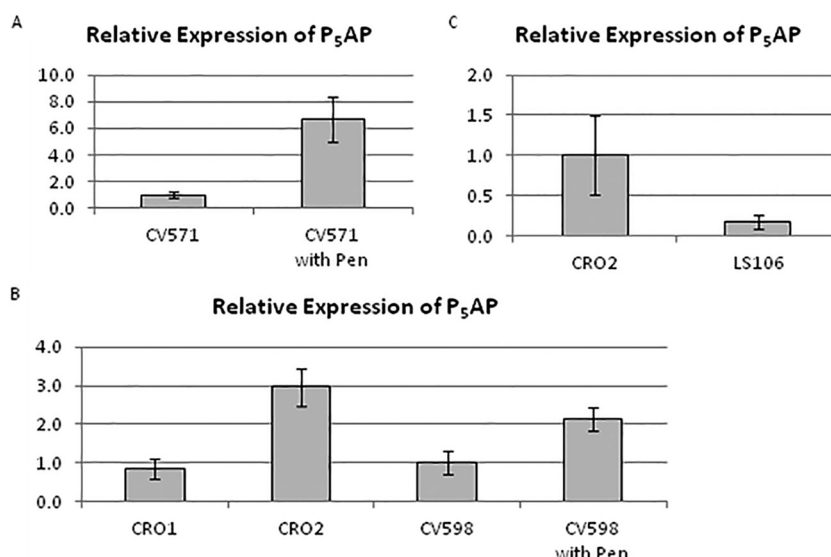


**FIG 2** Maximum likelihood tree.  $P_5AP$  homologues in *Firmicutes*. *E. faecium* Cro1 and *E. faecium* D344SRF are identical.  $P_5AP$  from *E. faecium* is closer to other enterococcal  $P_5AP$  sequences; interestingly, the closely related *Streptococcus*, *Lactococcus*, and *Leuconostoc* are highly divergent from the *Enterococcaceae*. The *Bacillales* all form a single branch with the exception of *Staphylococcus*. *E. faecium* Cro1 is highlighted in red. The *Lactobacillales* are shown in light blue. The *Bacillales* are shown in light magenta. *Staphylococcus* is shown in light yellow. *Clostridium* is shown in green.

coding sequence with the truncating mutation in a strain also expressing the native Stk confers cephalosporin resistance on our cephalosporin-susceptible class A Pbp mutants. Given that the truncation occurs in the extracellular portion of the molecule, we propose that it may result in a constitutive activation of the cytoplasmic kinase portion, with the resultant increase in the resistance to cephalosporins. In a similar fashion, expression of the wild-type *stpA-stk* in Cro2, which has a mutation in *stpA* predicted to truncate the protein to half its normal length, presumably eliminating its activity, was associated with the return of cephalosporin susceptibility, suggesting that the Cro2 mutation results in loss of function of StpA and cephalosporin resistance.

Similar to observations by Kristich and colleagues for *E. faecalis* (6), we observed a considerably higher level of cephalosporin resistance associated with the loss of StpA activity than with the presumably increased activity of Stk. They observed that a strain in which *ireP-ireK* was deleted exhibited increased susceptibility compared to the wild type and that a strain with an IreK with three active site threonines replaced by a phosphomimetic uncleavable by IreP exhibited high-level cephalosporin resistance even in the presence of active IreP, concluding that phosphorylation of IreK was essential for the expression of cephalosporin resistance. In

contrast, Western blot analysis of our Cro2 mutant using the Stk and the StpA antibodies detected a faint band corresponding to the size of the wild-type Stk and failed to demonstrate the presence of StpA (Fig. 1A). RT-PCR analysis of *stpA-stk* revealed the expected quantity of transcript (Fig. 1B), suggesting that translation of the *stk* message, which lies downstream of *stpA*, may depend upon translation of the terminal portion of *stpA*. The fact that the Cro2 mutant shows high levels of resistance to cephalosporins despite barely detectable levels of Stk on Western blot analysis suggests that the absence of StpA is the predominant factor in determining the level of cephalosporin susceptibility in *E. faecium*. As StpA has been shown to dephosphorylate Stk (8), the complete absence of StpA may result in a higher overall quantity of phosphorylated Stk, despite a considerably lower absolute quantity of protein (phosphorylated and unphosphorylated). It is also possible that, in contrast to *E. faecalis*, the primary role of Stk in the cephalosporin-resistant phenotype of class A Pbp-deficient *E. faecium* is through phosphorylation of StpA, which according to this model would serve to moderate the StpA phosphatase activity and thereby augment the resistance to cephalosporins. This model is at variance with the findings in *E. faecalis* (6), where *in vitro* experiments failed to demonstrate IreK phosphorylation of IreP. Alter-



**FIG 3** Expression of the *P<sub>5</sub>AP* gene is induced in response to exposure to penicillin or following acquisition of a nonsense mutation in *stpA*. Cultures were grown in BHI broth to mid-log phase, and penicillin was added at 12.5  $\mu$ g/ml (one-half of the MIC). After 30 min, cells were harvested and frozen for RNA preparation. Expression levels of 16S rRNA and *tufA* were used as references. Error bars indicate the standard error of the mean for biological duplicates. Results are representative of two independent experiments. (A) Relative gene expression of *P<sub>5</sub>AP* in ceftriaxone-susceptible double class A Pbp mutant CV571 in response to growth in penicillin. (B) Relative gene expression of *P<sub>5</sub>AP* in the Cro1 and Cro2 mutants and of ceftriaxone-susceptible triple class A Pbp mutant CV598 in response to growth in penicillin. (C) Relative gene expression of *P<sub>5</sub>AP* in the Cro2 mutant alone and transformed with the wild-type *stpA*-*stk*.

natively, StpA may dephosphorylate proteins other than Stk that are involved in cephalosporin resistance. It is clear that StpA plays an important role in expression of *P<sub>5</sub>AP*, since introduction of wild-type *stpA* into the Cro2 mutant reduces *P<sub>5</sub>AP* gene transcription and restores cephalosporin susceptibility. We are in the process of planning experiments to define the links between StpA activity and *P<sub>5</sub>AP* gene transcription.

When we originally decided to delete the class A Pbps of *E. faecium* and test the impact of those deletions on  $\beta$ -lactam resistance, our assumption was that Pbp5 could likely work with the previously described class A glycosyltransferases and that, lacking those glycosyltransferase partners, it would not be able to participate in cell wall synthesis. Since it has been established that Pbp5 is the sole Pbp that confers  $\beta$ -lactam resistance in *E. faecium* (strains lacking Pbp5 are fully susceptible to  $\beta$ -lactams) (3, 16), we anticipated that elimination of partner glycosyltransferases would yield susceptibility to a wide range of  $\beta$ -lactams. To our surprise, deletion of *ponA* and *pbpF* yielded strains that were susceptible to only a narrow range of cephalosporins (ceftriaxone and cefepime, which share a side chain) and retained resistance to other  $\beta$ -lactams. This susceptibility was reversible by exposure to penicillin (to which the strains are resistant), and resistant mutants were readily selected on cephalosporin-containing agar. In short,

the phenotype was not consistent with a model in which Pbp5 was unable to participate in peptidoglycan synthesis.

It has recently been reported (17) that the activity of ceftaroline against methicillin-resistant *Staphylococcus aureus* involves binding of ceftaroline to an allosteric site on low-affinity class B Pbp2a, triggering a conformation change that opens up the active site, allowing binding of a second ceftaroline molecule and inhibition of Pbp2a activity. These investigators reported that a similar allosteric site appeared to be present in *E. faecium* Pbp5. With this model in mind, we hypothesize that *E. faecium* Pbp5 has an allosteric site, capable of binding ceftriaxone and cefepime and creating a conformational change in the active site that allows binding and inhibition by a second cephalosporin molecule. We propose that the interaction between Pbp5 and other proteins, either class A Pbps (PonA or PbpF), *P<sub>5</sub>AP*, or a combination of these proteins, either precludes cephalosporin binding to the allosteric site or prevents the subsequent conformational change that normally occurs after allosteric binding. Deleting *ponA* and *pbpF* results in the interaction between cephalosporins and the allosteric site and hence susceptibility. Exposure to penicillin in these cephalosporin-susceptible strains results in increased expression of *P<sub>5</sub>AP*, which, we hypothesize, interacts with Pbp5 in a manner that precludes either allosteric binding or the resulting structural

**TABLE 3** MICs of *P<sub>5</sub>AP* clones in CV571

| Strain (genotype)                                    | Plasmid                                | Growth condition | MIC ( $\mu$ g/ml) of drug <sup>a</sup> : |      |      |       |
|--|--|------------------|--|------|------|-------|
|  |  |                  | PenG                                     | Amp  | Cro  | Chlor |
| CV571 ( $\Delta$ <i>pbpF</i> $\Delta$ <i>ponA</i> )  | None                                   | Uninduced        | 25                                       | 12.5 | 1.56 | 3.13  |
| CV571 ( $\Delta$ <i>pbpF</i> $\Delta$ <i>ponA</i> )  | None                                   | Induced          | 25                                       | 6.25 | 1.56 | 6.25  |
| LS108E ( $\Delta$ <i>pbpF</i> $\Delta$ <i>ponA</i> ) | pRIH113 ( <i>P<sub>5</sub>AP</i> gene) | Uninduced        | 50                                       | 50   | 6.25 | 3.13  |
| LS108E ( $\Delta$ <i>pbpF</i> $\Delta$ <i>ponA</i> ) | pRIH113 ( <i>P<sub>5</sub>AP</i> gene) | Induced          | 50                                       | 100  | 25   | 3.13  |

<sup>a</sup> Abbreviations: PenG, penicillin G; Amp, ampicillin; Cro, ceftriaxone; Chlor, chloramphenicol.

changes. Constitutive acquisition of cephalosporin resistance can occur in this setting through a null mutation in *stpA*, presumably through increased phosphorylation of a transcription factor for the P<sub>5</sub>AP gene.

At this time, we have no compelling data to suggest a specific activity for P<sub>5</sub>AP. It shares little similarity with proteins of known function. Our sequence searches retrieved homologous sequences in *Enterococcus* species, other *Enterococcaceae*, other *Lactobacillales* (*Lactobacillus*, *Streptococcus*, *Lactococcus*), *Bacillales* (*Staphylococcus* and *Listeria*), and *Clostridia* (*Clostridium*). Our search found that proteins containing DUF4097 (IPR025164) and DUF4098 (IPR025386) are not particularly conserved. The DUF4097/DUF4098-containing proteins in *Streptococcus* and *Lactococcus* are highly divergent from those in the enterococci. It is interesting that *Enterococcus* and *Listeria* are two species that are intrinsically resistant to cephalosporins, but their P<sub>5</sub>AP sequences share only 22.6% identity. While it is tempting to speculate that P<sub>5</sub>AP serves as a glycosyltransferase, there are no suggestions in the structure that it has such activity. Using the secondary structure prediction program PSIPRED, it was observed that residues 1 to 235 are mostly alpha helical while beta-sheets predominate in the C-terminal domain (residues 240 to 520). There are no obvious transmembrane regions or export signals, though its close association with Pbp5 strongly suggests that it acts in the extracellular milieu, since Pbp5 is predominantly an extracellular protein. Experiments are planned to better define the structure of P<sub>5</sub>AP and its interaction with Pbp5 to further refine our model for its involvement in *E. faecium* resistance to cephalosporins.

## MATERIALS AND METHODS

**Bacterial strains, growth media, chemicals, and microbiological techniques.** The strains used in this study are listed in Table 1. *E. faecium* cultures were grown in either brain heart infusion (BHI) broth (Fluka) or MM9YE minimal medium containing either glucose (2%) or ribose (0.2%) and rhamnose (2%) (1) at 37°C. *Escherichia coli* was grown in Luria-Bertani broth (LB) at 37°C. Concentrations of antibiotics for selection were as follows: kanamycin, 50 µg/ml (*E. coli*) or 1.5 µg/ml (*E. faecium*); chloramphenicol, 25 µg/ml (*E. coli*) or 10 µg/ml (*E. faecium*); rifampin, 100 µg/ml; fusidic acid, 50 µg/ml. All antibiotics and chemicals were obtained from Sigma-Aldrich unless otherwise stated. Disc diffusion assays were carried out by standard methods on BHI agar. Zone sizes were determined by visual inspection after 18 to 24 h of incubation. Microbroth MIC determinations were carried out by standard methods except that cells were grown in either BHI or MM9YE with either glucose (non-inducing medium) or rhamnose (inducing medium) (1). For phosphoproteins and Western blot assays, *E. faecium* cultures were grown in BHI broth until mid-logarithmic stage and then divided and treated with or without 10 µg/ml penicillin G (Sigma catalog no. P7794) for 1 h at 37°C with shaking at 200 rpm. Cells were harvested from 50-ml culture volumes, and cell pellets were stored at -80°C. For the TAP method, cells were grown in BHI with or without 10 µg/ml penicillin until mid-log stage. Cells were washed in phosphate-buffered saline, and pellets were stored at -80°C.

**Isolation of *E. faecium* ceftriaxone-resistant clones.** The parental strains CV558 and CV598 were streaked on BHI agar from frozen stock and grown overnight. McFarland standards of 0.5 were made in BHI broth and spread onto BHI plates. A single ceftriaxone disc (30 µg) was applied to each plate, which was incubated for 48 h at 37°C. Zones of inhibition around the antibiotic discs were observed, with resistant colonies growing in the zone. Multiple colonies from the ceftriaxone zones were selected and streaked for single colonies on BHI agar. A single colony from each streak was selected and restreaked for a total of 10 passages. Isolates were then retested by disc diffusion, and a resistant isolate was

selected for whole-genome sequencing. A second mutant was isolated as before and screened by sequencing the *stpA* and *stk* genes.

**Genome sequencing and analysis to identify mutations in ceftriaxone-resistant strains.** Genomic DNA was extracted from overnight cultures. The genomic DNA libraries were constructed using a previously described method (18) with minor modifications. Genomic libraries were pooled for sequence analysis on one lane of an Illumina HiSeq 2000 II sequencing system. Assembly was accomplished by alignment with the *E. faecium* Aus0004 genome (19). Genomics comparisons between the sequenced strains were used to identify insertions, deletions, and single point mutations that may be responsible for the observed resistance profiles. High-confidence mutations in coding sequences that were identified initially were confirmed by Sanger sequencing of PCR products.

**Molecular biology techniques.** DNA-modifying enzymes and kits were purchased from New England Biolabs, unless otherwise noted. Primers were obtained from Sigma. All PCRs were carried out using Phusion high-fidelity polymerase from New England Biolabs, according to the manufacturer's directions. Plasmid preparations were carried out using Qiagen midiprep kits. Enterococcal genomic DNA was made using the Qiagen DNeasy kit.

**Construction of plasmids and transfer to *E. faecium*.** Due to the homology seen to *ireK* identified in *E. faecalis* (1), *stk* from Cro1 was chosen for further analysis. Mutant and wild-type versions of the *stk* gene, along with the upstream *stpA* gene, were cloned by PCR into the *E. coli*/Gram-positive shuttle vector pTCV-lac (20) behind the rhamnose-inducible promoter from pCJK96 by standard methods. This plasmid was transformed into *E. coli* SM10 and mated into *E. faecium* in BHI broth. Transconjugants were selected with kanamycin and either chloramphenicol (for CV558 and CRO2) or rifampin plus fusidic acid (for D344S).

The tandem affinity purification (TAP)-*pbp5* fusion was constructed by cloning a PCR product of Pbp5 coding sequence plus ~800 bp upstream into pBS1479 (13). After sequence confirmation, the fusion protein was transferred into pTCV-lac. This plasmid was electroporated into CV571, selecting with kanamycin. For the TAP construct with the Tap tag alone, the Pbp5 promoter region (~800 bp) was independently cloned into pBS1479. The SapI/SmaI fragment was transferred into pTCV-lac as before.

The P<sub>5</sub>AP overexpression plasmid was constructed by cloning the coding sequence into SmaI/SalI sites of pTCV-lac (eliminating *lacZ* and *ermB*) behind the rhamnose-inducible promoter from pCJK96. This plasmid was electroporated into CV571.

**Gene expression studies.** Relevant strains were grown with shaking at 37°C to an A<sub>600</sub> of between 0.4 and 0.5. At that point, aliquots had penicillin added at a final concentration of half the MIC value and were grown for a further half hour with shaking. Cells were broken open with glass beads (Lysing Matrix B; MP Biomedical) using a mini-BeadBeater (Bio-Spec), and RNA was purified using a Qiagen RNeasy minikit. Quantitative reverse transcriptase PCR was carried out using a Bio-Rad iQ universal SYBR one-step kit and a CFX98 real-time PCR cyclor. Relative gene expression was calculated using the Pfaffl method (24) and normalized relative to expression of 16S rRNA and *ith* mRNA.

**Microarray analyses.** In triplicate, total bacterial RNA was isolated from *E. faecium* strain CV571 following a 30-min treatment with 10 µg/ml penicillin or no drug. A total of 10 µg RNA from each sample was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The resulting cDNA was purified using QIAquick PCR purification kits (Qiagen, Germantown, MD), fragmented with DNase I (Ambion, Carlsbad, CA), and then 3' biotinylated using Bioarray terminal labeling kits (Enzo Life Sciences, Farmingdale, NY). A total of 1.5 µg of labeled material from each sample was hybridized to custom-made *E. faecium* Affymetrix GeneChips (part number PMDefcma520788F) that contained a total of 4,395 probe sequences representing 3,898 open reading frames and 497 intergenic *E. faecium* annotated GenBank entries at the time of array design. GeneChips were then processed according to the



manufacturer's recommendations for prokaryotic arrays (Affymetrix, Santa Clara, CA). Differentially expressed transcripts were identified as RNA species that demonstrated a 1.5-fold increase or decrease in expression in penicillin-challenged CV571 cells in comparison to mock treatment ( $t$  test,  $P = 0.05$ ).

**Preparation of protein lysates for SDS-PAGE and phosphoprotein analysis.** All chemicals were obtained from Sigma unless otherwise specified. Cell pellets were washed twice in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl buffer, and resuspended at 50 mg/ml in lysis buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl with 5 mM EDTA, 1× HALT protease inhibitor cocktail, and 0.5× HALT phosphatase inhibitor cocktail (Thermo Scientific). Cells were processed 4 times for 30 s each in Lysing Matrix B tubes using the mini-BeadBeater (BioSpec Products), lysates were clarified, and protein concentrations were determined using the 2-D Quant kit (GE Healthcare). The lysates were precipitated according to the Invitrogen Pro-Q Diamond phosphoprotein gel stain (Molecular Probes) recommendations using the methanol-chloroform protocol. The PeppermintStick phosphoprotein molecular weight standards (Molecular Probes) were diluted and treated as recommended and used for both molecular weight markers and positive and negative controls for the phosphoprotein gel stain. Protein samples solubilized in 1× LDS sample buffer (Invitrogen) were separated using Invitrogen NuPAGE 4 to 12% Bis-Tris 1-mm gels with MES SDS (2-morpholinoethanesulfonic acid sodium dodecyl sulfate) running buffer and NuPAGE antioxidant for 40 min at 200 V.

The Invitrogen Pro-Q Diamond protocol for in-gel staining of phosphoproteins was followed except for a modification using 3-fold-diluted Pro-Q Diamond phosphoprotein stain, and the incubation step increased to 120 minutes per the work of Agrawal and Thelen (21). The gel was imaged using the Bio-Rad ChemiDoc XRS Imager with the trans-UV setting at 302-nm excitation and the standard emission filter with a range of 535 to 640. The gel was further analyzed by staining with SYPRO Ruby protein gel stain using the Rapid Protocol method as described in the SYPRO Ruby protocol (Molecular Probes). Duplicate 4 to 12% Bis-Tris NuPAGE gels were run for Western blot assays using MagicMark XP Western protein standard (Invitrogen) for molecular weight estimation.

**Western blot analysis.** After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes using the iBlot (Invitrogen) dry blotting system. Polyclonal rabbit anti-Stk and anti-StpA antibodies were used at recommended dilutions of 1/2,000 and 1/1,000, respectively (8). A Bis-Tris detergent-buffered saline solution with Hammersten casein was used for the block and antibody dilutions (Invitrogen; Western Breeze blocker/diluent). Goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Invitrogen) was used at a 1/10,000 dilution, and the proteins were detected with Clarity Western enhanced chemiluminescence (ECL) blotting substrate (Bio-Rad).

**Tandem affinity purification.** The C terminus of the *pbp5* gene in strain CV571 was epitope tagged with the TAP tag, consisting of a calmodulin binding peptide and a protein A moiety separated by a tobacco etch virus (TEV) cleavage site, allowing for a two-step affinity purification of protein complexes with minimal background contaminants. *E. faecium* strains LS002 and LS107, carrying the Pbp5 fusion with the TAP tag and the TAP tag alone, respectively, were used for the TAPs. Cell pellets were resuspended in lysis buffer containing 10 mM Tris-HCl (pH 8), 100 mM NaCl, 0.1% NP-40, and HALT protease inhibitors (Thermo Scientific) and disrupted by bead beating in a BioSpec BeadBeater, and the lysate was clarified by centrifugation. The TAP was performed according to the protocol of Puig et al. (22) with slight modifications. IgG-Sepharose binding was performed in a 50-ml conical tube with 600  $\mu$ l of IgG Sepharose 6 Fast Flow bead suspension, and the tube was incubated overnight at 4°C. The unbound fraction was removed by centrifugation, and the resin was resuspended in 10 ml of IgG-Sepharose binding buffer and transferred into an 0.8- by 4-cm Poly-Prep column (Bio-Rad). The resin was subsequently washed with binding buffer followed by TEV cleavage buffer (10 mM

Tris-HCl, pH 8, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, and 1 mM dithiothreitol). Cleavage was performed in the column with 300 units of ProTEV Plus (Promega) in TEV cleavage buffer rotating overnight at 30°C. The eluate was collected by gravity flow and treated according to the protocol in the work of Puig et al. (22). Calmodulin affinity purification of the TEV-cleaved material was performed at 4°C for 2 h with rotation in a second column using 600  $\mu$ l of calmodulin affinity resin suspension (Agilent Technologies). Unbound material was removed by gravity flow followed by two successive washes with calmodulin binding buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole) containing first 0.1% NP-40 and then 0.02% NP-40. The protein complexes were eluted with calmodulin elution buffer containing 10 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM EGTA, and 10 mM beta-mercaptoethanol after a 5-min incubation at 4°C.

The eluted protein complexes were precipitated with trichloroacetic acid (TCA) according to the TCA-acetone protocol obtained from the Taplin Mass Spectrometry Facility and submitted for mass spectrometric processing and analysis.

**Phylogenetic analysis of P<sub>5</sub>AP.** To identify P<sub>5</sub>AP homologues, we used the HMMER online server (<http://www.ebi.ac.uk/Tools/hmmer/search/phmmer>) to search for proteins with the DUF4097 and DUF4098 domains. We used default values and used the UniProtKB database. The HMMER search against UniProtKB retrieved 795 proteins, 781 from *Bacteria* and 705 of those from *Firmicutes*, mostly concentrated in the *Bacilli* (558 proteins; see Table S3 in the supplemental material). We selected representative sequences from *Enterococcus*, other *Lactobacillales*, *Bacillales*, and two *Clostridiales* for phylogenetic comparison. For phylogenetic analyses, protein sequences were aligned using Clustal Omega. Phylogenetic analyses were then carried out using PhyLM online (23) using the LG substitution model, NNI tree searching, and aLRT SH-Like branch support. The resulting tree and matrix were visualized with Geneious 8.1.5.

**Microarray data accession number.** The GeneChip format and corresponding data files were deposited in the NCBI Gene Expression Omnibus repository under accession number GSE77436.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02188-15/-/DCSupplemental>.

Figure S1A, PDF file, 0.3 MB.  
Figure S1B, PDF file, 0.2 MB.  
Figure S2, PDF file, 0.3 MB.  
Figure S3, PDF file, 0.2 MB.  
Table S1, DOC file, 0.03 MB.  
Table S2, XLSX file, 0.04 MB.  
Table S3, XLSX file, 0.1 MB.  
Table S4, XLS file, 0.2 MB.

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